Role of EP2 and EP4 receptor-selective agonists of prostaglandin E_2 in acute and chronic kidney failure

S Vukicevic[1], P Simic[1], F Borovecki[1], L Grgurevic[1], D Rogic[2], I Orlic[1], WA Grasser[3], DD Thompson[3] and VM Paralkar[3]

[1]*Laboratory of Mineralized Tissues, Department of Anatomy, Zagreb Medical School, University of Zagreb, Zagreb, Croatia;* [2]*Central Biochemical Laboratory, Clinical Hospital Center Rebro, Zagreb, Croatia and* [3]*Pfizer Global Research and Development, Groton Laboratories, Groton, Connecticut, USA*

We tested the efficacy of three selective agonists of prostaglandin E_2 (PGE_2) receptor, EP2 (CP-536,745-01), EP2/4 (CP-043,305-02), and EP4 (CP-044,519-02), in two models of acute and chronic kidney failure. In the nephrotoxic mercury chloride ($HgCl_2$) rat model of acute kidney failure systemically administered EP4 agonist reduced the serum creatinine values and increased the survival rate. Although the EP2 or the EP2/4 agonist did not change the serum creatinine values, the EP2 receptor agonist increased the survival rate. Histological evaluation of kidneys from EP4-treated rats indicated less proximal tubular necrosis and less apoptotic cells. In a rat model of chronic renal failure, the three receptor agonists decreased the serum creatinine and increased the glomerular filtration rate at 9 weeks following therapy. Kidneys treated with the EP4 agonist had less glomerular sclerosis, better preservation of proximal and distal tubules and blood vessels, increased convoluted epithelium proliferation and less apoptotic cells. Nephrectomy had no influence on the expression of the EP4 receptor, whereas EP2 receptor expression was reduced by 50% and then corrected following treatment with EP2 and EP2/4 receptor agonists. These findings suggest that PGE_2 has an important role in acute kidney failure via the EP4 receptor, whereas in chronic kidney failure both EP2 and EP4 receptors are equally important in preserving the progression of chronic kidney failure. Thus, agonism of EP2 and EP4 receptors may provide a basis for treating acute and chronic kidney failure.

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Correspondence: *S Vukicevic, Laboratory of Mineralized Tissues, Department of Anatomy, Zagreb Medical School, University of Zagreb, Salata 11, 10000 Zagreb, Croatia. E-mail: vukicev@mef.hr*

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Acute and chronic renal failures are directly responsible for high rate of mortality, significant morbidity, and high medical costs.[1,2] Acute renal failure (ARF) is a very morbid and costly disorder with a severe proportion of patients progressing to end-stage renal disease requiring dialysis. Despite the advances of supportive care, the mortality has remained high (40–80%).[3] Although the damaged kidney is capable of complete repair and regeneration after acute injury, the successful treatment of patients with ARF who require dialysis remains one of the greatest challenges facing nephrology today.[4,5] The mechanisms controlling the cascade of cellular migration, growth, and proliferation undoubtedly comprise a number of autocrine and paracrine growth factors.[6,7] Several novel compounds have proved effective in the treatment of experimental ARF, but there has been little success in human clinical trials, including synthetic atrial natriuretic peptide, recombinant human insulin-like growth factor-1, calcium channel antagonists, thyroxine, and endothelin receptor antagonists.[8]

Chronic renal failure (CRF) is another entity, which progresses to end-stage renal failure, independently of the initial pathogenic mechanism. The early stages of renal injury involve compensatory renal growth associated with cellular hypertrophy and hyperplasia.[9,10] The infiltration of platelets, lymphocytes, and monocytes into the glomeruli and interstitium causes the progression of renal scarring.[11,12] This scarring process then leads to a progressive damage of glomerular and tubular cells, and eventually causes glomerular sclerosis and tubular atrophy. In end-stage renal disease, renal cells are replaced with fibrous tissue contributing to the sclerotic changes observed in the glomeruli and the interstitium.[11,12] When the glomerular filtration rate (GFR) declines to less than 10% of normal values (5–10 ml/min), the renal failure will rapidly progress to cause death unless the patient receives renal replacement therapy (i.e. chronic hemodialysis, continuous peritoneal dialysis, or kidney transplantation) or alternatively a therapy that delays the progression of the CRF.

At present, there is no specific therapy for chronic renal disease, and treatment with growth factors may ameliorate the progression of the kidney disease.[13] A potential treatment

for ARF and CRF could be PGE_2 which has been shown to have multiple biological effects in many tissues including kidney. PGE_2 plays a major role in the normal renal physiology including production of renin, regulation of GFR, and tubular re-absorption of salt and water.[14] Similarly, in disease states such as diabetic nephropathy, PGE_2 synthesis is elevated. PGE_2 plays an important role in maintaining the blood flow especially during conditions of enhanced vasoconstrictor activity. Furthermore, PGE_2 inhibits proliferation of cultured mesangial cells *in vitro*,[15] and given the diversity of PGE_2 actions in the kidney it is attractive to speculate a therapeutic role for PGE_2 in chronic and/or acute kidney failure. However, owing to side effects that include diarrhea, lethargy, and flushing, PGE_2 is an unacceptable therapeutic option.

The pharmacological activity of PGE_2 is carried out via four different cell surface receptor subtypes: EP1, EP2, EP3, and EP4. Of these four receptors, three are involved in modulation of cyclic adenosine 5′-monophosphate (cAMP) levels (EP2 and EP4 increase cAMP levels, EP3 reduces cAMP levels). The fourth receptor, EP1, is involved in regulating intracellular calcium levels. All four of the EP receptors have been localized in the kidney and are responsible for distinct actions of PGE_2. The EP1, EP3, and EP4 receptors are present in the collecting duct. EP4 has also been shown to be expressed in the glomerulus and EP2 mRNA has been localized to the outer and inner medulla of the rat kidney.[16,17] Despite the well-known activities of PGE_2 in maintaining the kidney function and localization of various prostanoid receptors in the kidney, the precise role of their agonists or antagonists as therapeutic options remains poorly understood. Recently, an EP1 selective antagonist has been demonstrated to be effective in prevention of diabetic nephropathy in a streptozotocin-induced rat model.[18] We initiated a discovery effort to identify the role of EP2 and EP4 receptor-selective agonists in rat models of acute and chronic kidney disease.

In the present study, we have used three different selective agonists of the PGE_2 receptors to assess the ability of agonists of receptor subtypes to treat rats with acute and chronic renal failure. Of the three receptor-specific agonists tested, CP-043,305-02 was selective for both the EP2 and EP4 receptors, CP-044,519-02 is an EP4-specific agonist, and CP-536,745-01 is an EP2-specific agonist. In this report, we show that CP-044,519-02, a highly selective and potent functional EP4 receptor agonist, is efficacious in a rat model of acute renal failure, whereas both EP2 and EP4 receptor agonism prevents the progression of chronic renal failure.

RESULTS

EP2 and EP4 receptor agonists

We hypothesized that specific PGE_2 receptor subtype agonists (Table 1) would have a positive impact on renal damage in the rat and be devoid of severe side effects of PGE_2. To test our hypothesis, we sought EP2, EP4, and EP2/4 receptor-selective agonists. CP-043,305-02, CP-044,519-02, and CP-536,745-01 bound with high affinity and were selective and potent full agonists of the EP receptor, as measured by their ability to increase intracellular cAMP levels to the same levels as treatment with PGE_2 (Table 1).

Table 1 | PGE_2 receptor subtype agonists

	EP2 EC_{50} (nM)	EP4 EC_{50} (nM)
CP-536,745-01 (EP2)	1.2	
CP-043,305-02 (EP2/4)	384	8.6
CP-044,519-02 (EP4)		2.5

Mercury chloride model of acute renal failure

Serum creatinine (Cr) values of control rats increased 7.5-fold on day 1 and 3 following $HgCl_2$ injection (Figure 1a). In rats treated with 10 mg/kg of an EP4 (CP-044,519-02) receptor agonist, Cr values were significantly lower on days 1–3 (Figure 1a). A dose of 1 mg/kg EP4 receptor agonist was less effective, showing a statistical significance only on day 2 following mercury chloride ($HgCl_2$) injection. At 6 days after $HgCl_2$ application, there were no differences between treatment groups due to the low survival rate of control animals (Figure 1a). Dynamic changes of blood urea nitrogen showed a similar trend as serum Cr value. Animals treated with 10 mg/kg of EP4 had lower blood urea nitrogen on days 1 and 2 as compared to control animals, whereas rats treated with 1 mg/kg had lower values only on day 1 (data not shown). Therapy with the EP2 (CP-536,745-01) and EP2/4 (CP-043,305-02) receptor agonist had no effect on serum Cr values (Figure 1b and c). At termination on day 6 following $HgCl_2$ administration, 90–95% of rats treated with both 1 and 10 mg/kg EP4 survived, whereas 85% of the rats treated with EP2 survived (Figure 1b). About 73% of rats survived in the group treated with 10 mg/kg of EP2/4 receptor agonist, whereas 1 mg/kg EP2/4 was ineffective (Figure 1c). On the contrary, only 40% vehicle-treated control rats survived at day 6 following $HgCl_2$ injection (Figure 1).

Based on the functional remnant kidney parameters, we next analyzed the effect of the EP4 receptor agonist on the renal histopathology and apoptosis. The cellular necrosis of the proximal tubules was moderate in the EP4 receptor agonist-treated rats, whereas EP2 and EP2/4 receptor agonists were less effective or had no effect (Table 2). However, no histopathological changes were found in the glomeruli (Figure 2). There was a lower number of apoptotic cells in the kidneys treated with the EP4 receptor agonist at the termination of the experiment, suggesting that apoptosis is an important event in the nephrotoxic kidney damage and that prevention and recovery are best pronounced in the EP4 receptor agonist-treated rats (Table 3). These results suggest that EP4 receptor agonism had an effect on the biochemical renal serum parameters, correlated with significantly improved histopathological findings as compared to similar analyses of kidney sections treated with EP2 and EP2/4 receptor agonists (Tables 2 and 3).

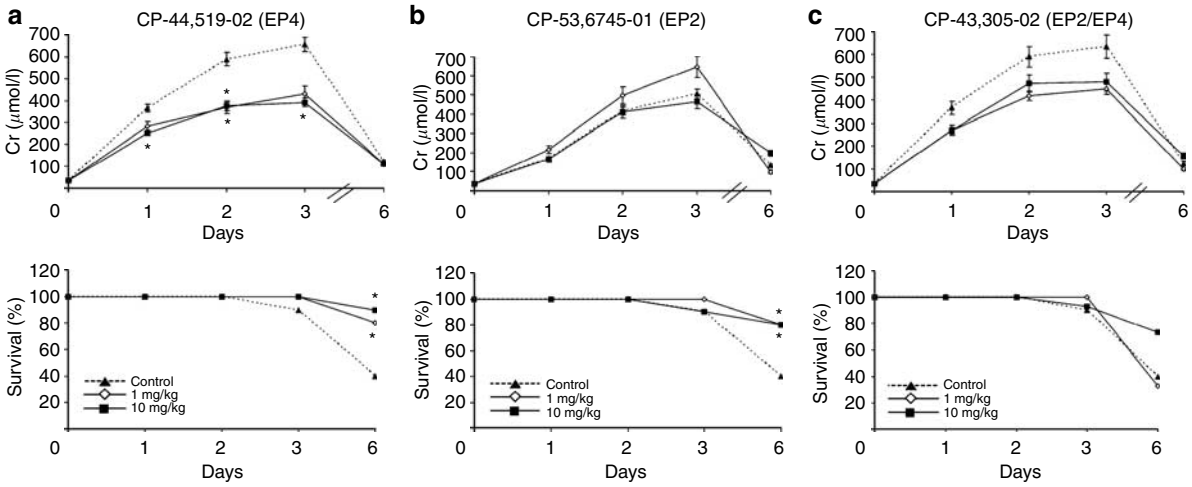

Figure 1 | Therapeutic effect of (a) CP-044,519-02 (EP4), (b) CP-536,745-01 (EP2), and (c) CP-043,305-02 (EP2/4) on serum Cr and survival in the $HgCl_2$ nephrotoxic rat model. EP4 receptor agonist decreased serum Cr and increased the survival rate. EP2 and EP2/4 receptor agonists had no effect on serum Cr values; EP2 increased the survival rate; only the dose of 10 mg/kg EP2/4 increased the survival. Results are mean ± s.e.m. of 10 rats per treatment group. *$P < 0.05$ with respect to $HgCl_2$-control rats. Moribund rats were killed to prevent suffering (see Materials and Methods).

Table 2 | Proximal tubular necrosis in $HgCl_2$ control, CP-044,519-02 (EP4)-, CP-536,745-01 (EP2)-, and CP-043, 305-02 (EP2/4)-treated rats

Treatment groups	Scores for the severity of necrosis in proximal tubules
Control vehicle	9.2 ± 0.64
CP-044,519-02 (1 mg/kg)	4.6 ± 0.31*
CP-044,519-02 (10 mg/kg)	3.9 ± 0.26*
CP-536,745-01 (1 mg/kg)	8.1 ± 0.92
CP-536,745-01 (10 mg/kg)	6.9 ± 0.42
CP-043,305-02 (1 mg/kg)	10.9 ± 2.4
CP-043,305-02 (10 mg/kg)	6.3 ± 0.72

Results are mean ± s.e.m. of 10 kidneys (three sections each) per treatment group. *$P < 0.05$ with respect to $HgCl_2$-control rats.

Table 3 | Effect of CP-044,519-02 (EP4), CP-536,745-01 (EP2), and CP-043,305-02 (EP2/4) on apoptosis in rats with ARF

Treatment groups	Number of apoptotic cells per slice
Control	1387 ± 568
CP-044,519-02 (1 mg/kg)	895 ± 322*
CP-044,519-02 (10 mg/kg)	746 ± 214*
CP-536,745-01 (1 mg/kg)	1480 ± 506
CP-536,745-01 (10 mg/kg)	1120 ± 301
CP-043,305-02 (1 mg/kg)	1350 ± 240
CP-043,305-02 (10 mg/kg)	889 ± 112*

Results are mean ± s.e.m. of 10 kidneys (three sections each) per treatment group. *$P < 0.05$ with respect to $HgCl_2$-control rats.
Abbreviation: ARF, acute renal failure.

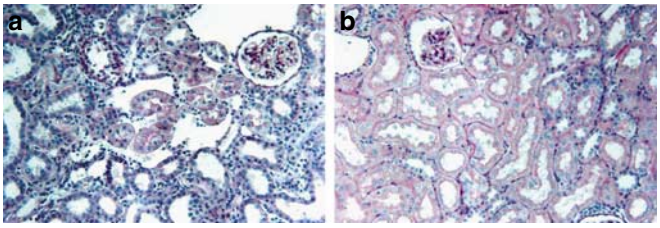

Figure 2 | Effect of CP-044,519-02 (EP4) on renal histology (periodic acid-Schiff stain) in rats subjected to $HgCl_2$ injection. The cellular necrosis of the proximal tubules was decreased in the EP4 receptor agonist-treated rats and no histopathological changes were found in the glomeruli. Comparison of typical histology from animals treated with vehicle (**a**, original magnification × 25) or CP-044,519-02 (**b**, original magnification × 25) at day 6 of therapy.

5/6 nephrectomy model of chronic renal failure

Serum Cr concentrations increased 70% in Nx animals throughout the duration of the experiment (Figure 3). In contrast, animals receiving EP4, EP2, and EP2/4 receptor agonists once a week had lower serum Cr values at 9 and 11 weeks following the beginning of therapy (Figure 3a). In an another experiment, Nx rats injected with EP4 receptor agonist three times a week had lower serum Cr values 8 and 11 weeks following the beginning of therapy (Figure 3b), and did not show serious side effects that include diarrhea, lethargy, and increased body temperature. Both, 1 and/or 3 weekly administrations of EP4 receptor agonist were equally effective in delaying the progression of the disease (Figure 3b). GFR was 41% higher at 8 weeks following the beginning of therapy in rats receiving the EP4 receptor agonist three times a week, whereas animals dosed once weekly had GFR increased 45%, 11 weeks following treatment (Table 4).

EP4 receptor agonist-treated rats had reduced glomerular sclerosis, more viable glomeruli, less tubulointerstitial injury, and better preservation of proximal and distal tubule structures as compared to control Nx rats (Table 5, Figure 4). Similar histopathologic prevention of the progression of kidney damage was detected in animals treated with EP2 and EP2/4 receptor agonists (data not shown). The immunohistochemical analysis of peritubular capillary-derived

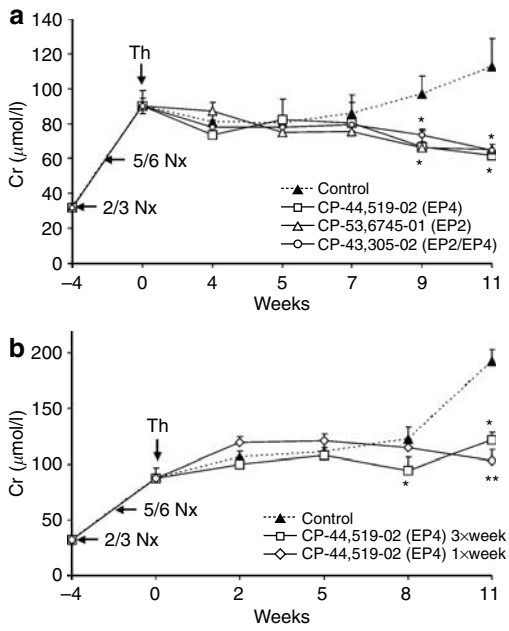

Figure 3 | Therapeutic effect of 1 mg/kg of CP-044,519-02 (EP4), CP-536,745-01 (EP2), and CP-043,305-02 (EP2/4) once a week and CP-044,519-02 (EP4) once and three times a week on serum Cr in a 5/6 Nx rat model of chronic renal failure. (**a**) All three receptor agonists were effective in reducing serum Cr values of Nx rats 9 and 11 weeks following Nx. (**b**) Both, 1 and/or 3 weekly administrations of EP4 receptor agonist were equally effective in delaying the progression of the disease. Results are presented as mean ± s.e.m. of (**a**) 25 rats per treatment group in and (**b**) 12 rats per treatment groups in. *$P<0.05$ vs control; **$P<0.005$ vs control. 2/3 Nx and 5/6 Nx indicates surgical nephrectomies as described in Materials and Methods; Th indicates the beginning of therapy.

Table 4 | Effect of CP-044,519-02 (EP4) on GFR (ml/min/g) in rats with CRF

	GFR (ml/min/g)			
	Weeks of therapy			
Therapy	2	5	8	11
Control vehicle	0.55 ± 0.23	0.62 ± 0.23	0.50 ± 0.16	0.35 ± 0.10
CP-044,519-02 (10 μg/kg) 3 × /week	0.51 ± 0.16	0.68 ± 0.16	0.85 ± 0.41[a]	0.63 ± 0.37[a]
CP-044,519-02 (10 μg/kg) 1 × /week	0.50 ± 0.19	0.55 ± 0.10	0.57 ± 0.30	0.64 ± 0.43[a]

Results are mean ± s.e.m. of 10 rats per treatment group.
*$P<0.05$ with respect to Nx-control rats.
Abbreviations: GFR, glomerular filtration rate; Nx, nephrectomy.

Table 5 | Morphological lesions of Nx control and CP-044, 519-02 (EP4)-treated rats in CRF

	Group		
	Vehicle	CP-044,519-02 1 × /week	CP-044,519-02 3 × /week
Tubulointerstitium	2.82 ± 0.23	1.36 ± 0.30[b]	1.00 ± 0.28[b]
Tubular dilatation/athrophy	3.0 ± 0.28	1.25 ± 0.30[b]	1.00 ± 0.32[b]
Interstitial fibrosis	2.33 ± 0.33	1.25 ± 0.30[a]	1.20 ± 0.26[a]
Inflammatory cells	2.67 ± 0.38	1.50 ± 0.18[a]	1.00 ± 0.22[a]
Glomeruli	2.82 ± 0.19	1.14 ± 0.33[b]	0.82 ± 0.19[b]
Glommerular sclerosis	2.67 ± 0.38	1.25 ± 0.16[a]	0.80 ± 0.14[a]
Microaneurysms	2.00 ± 0.48	0.75 ± 0.30	0.60 ± 0.17[a]
Absence of viable glomeruli	2.33 ± 0.32	1.00 ± 0.37[a]	0.60 ± 0.04[a]

Morphological lesions were quantified as described in the Materials and Methods section. Two independent observers without significant over- or underestimation between them analyzed kidneys of 12 animals per treatment group. The results are presented as mean ± s.e.m. of 10 kidneys (three sections each) per treatment group.
[a]$P<0.05$, [b]$P<0.005$ with respect to Nx-control rats.
Abbreviation: Nx, nephrectomy.

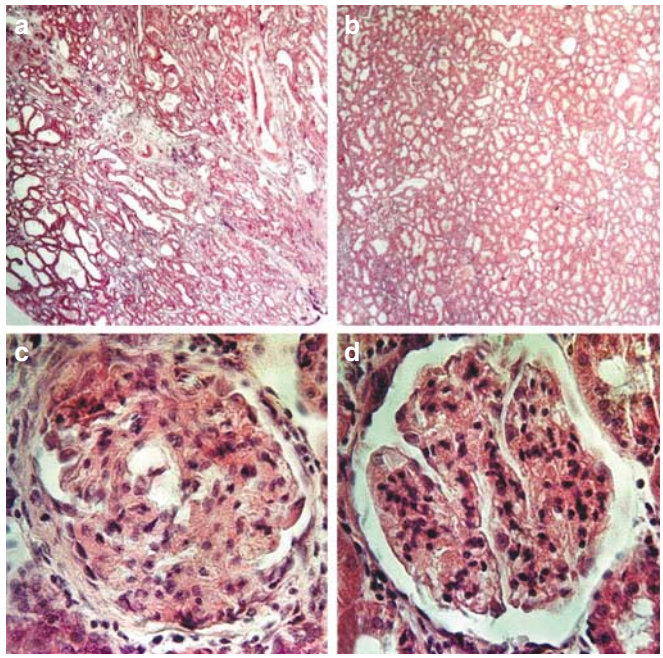

Figure 4 | Effect of CP-044,519-02 (EP4) on renal histology (hematoxylin and eosin) in rats subjected to 5/6 Nx. EP4 receptor agonist-treated rats had reduced glomerular sclerosis, more viable glomeruli, less tubulointerstitial injury, and better preservation of proximal and distal tubule structures as compared to control Nx rats. A typical histology from animals treated with a vehicle (original magnifications: **a**, × 12.5; **c**, × 50) or CP-044,519-02 (**b**, × 12.5, **d** × 50) at 11 weeks of therapy.

smooth muscle cells revealed that approximately fourfold more cells in EP4-treated kidneys expressed smooth muscle α-actin, suggesting that EP4 receptor agonist supports the maintenance of a vascular smooth muscle cell phenotype (Figure 5a and b).

Cell proliferation evaluated by proliferating cell nuclear antigen (PCNA) staining indicated an increase in the proximal tubule cell proliferation in EP4 receptor agonist-treated rats (Figure 5c and d). EP4 also attenuated the expression of intracellular adhesion molecule-1, an important molecule mediating the progress and intensity of renal necrosis and subsequent fibrosis (Figure 5e and f). Mac CD 68 staining detected an accumulation of macrophages in vehicle-treated rats. In contrast, rats treated with EP4 receptor agonist had

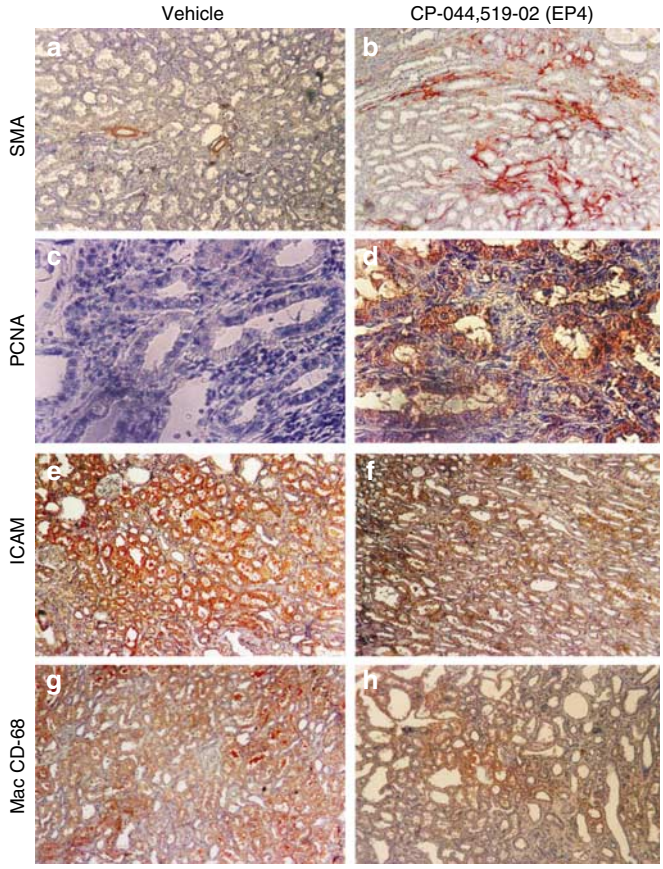

Figure 5 | Immunohistochemical staining of smooth muscle α-actin, proliferating cell nuclear antigen, intercellular cell adhesion molecule, and macrophage-associated antigen CD 68. Comparison of (**a, b**) smooth muscle α-actin, (**c, d**) PCNA, (**e, f**) intracellular adhesion molecule and (**g, h**) Mac staining of (**a**, **c**, **e, g**) vehicle and (**b**, **d**, **f, h**) CP-044,519-02-treated kidneys at 11 weeks of therapy. Fourfold more cells in EP4-treated kidneys expressed smooth muscle α-actin, maintaining vascular smooth muscle cell phenotype. Cell proliferation evaluated by PCNA staining indicated an increase in proximal tubule cell proliferation in EP4 receptor agonist-treated rats. EP4 also attenuated the expression of intracellular adhesion molecule -1, mediating renal fibrosis. Mac CD 68 staining detected ninefold less macrophages accumulated in the remaining kidney parenchyme of rats treated with the EP4 receptor agonist. Original magnification × 12.5.

approximately ninefold less macrophages accumulated in the remaining kidney parenchyme (Figure 5g and h).

EP2 and EP4 receptor expression

The effect of EP4, EP2, and EP2/4 receptor agonists on EP2 and EP4 mRNA was evaluated by real-time polymerase chain reaction in kidneys of 5/6 Nx control rats (Figure 6a) and in those treated with all three PGE_2 receptor agonists. Following nephrectomy (Nx), the EP2 receptor expression was reduced by 50% as compared to sham animals, however, treatment with the EP2/4 receptor agonist brought the EP2 expression levels back to sham values (Figure 6a). EP4 expression level did not change following Nx and treatment with EP2/4 had a slight effect on the EP4 gene expression (Figure 6a). At the end of the therapy, there was a slight elevation in EP2 and EP

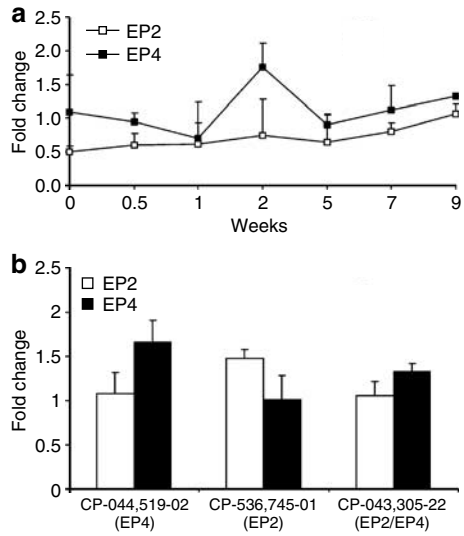

Figure 6 | EP2 and EP4 receptors expression during the therapy in the kidneys of 5/6 Nx rats treated with CP-043,305-02 (EP2/4) (a), and the end of the therapy in the kidneys of 5/6 Nx rats treated with CP-044,519-02 (EP4), CP-536,745-01 (EP2), and CP-043,305-22 (EP2/4). Following Nx, the EP2 receptor expression was reduced by 50% (0.5-fold change) as compared to sham animals ($P<0.05$). Treatment with the EP2/4 receptor agonist brought the EP2 expression levels back to sham values (**a**). EP4 expression level did not change following Nx and treatment with EP2/4 had a slight effect on the EP4 gene expression as compared to sham animals (**a**). At the end of the therapy, there was a slight elevation in EP2 and EP4 receptor expression in rats treated with all three PGE_2 receptor agonists as compared to sham animals (**b**). All data are represented as fold change to gene expression of sham animals ± s.e.m. (represented by 1.0 on the *y*-axis).

4 receptor expression in rats treated with all three PGE_2 receptor agonists as compared to sham animals (Figure 6b).

DISCUSSION

PGE_2 is ubiquitously present in animals and humans and has potent responses in a number of organ systems. Data in pre-clinical animal models and humans suggest that PGE_2 plays a crucial role in normal as well as pathological renal function.[19–25] The present study indicates that CP-044, 519-02, a selective agonist of the PGE_2 EP4 receptor, reduces nephrotoxic injury and increases the survival rate of animals with ARF. Biochemical serum parameters indicate that systemically administered EP4 receptor agonist limits the extent of injury and/or speeds up the recovery of injured tubular cells. The same trends of both parameters strongly indicate the beneficial effect of the EP4 receptor agonist in the $HgCl_2$-caused kidney damage. $HgCl_2$ exerts its toxic effects on kidney cells through a variety of mechanisms, with the principal target being the S3 segment of the proximal tubules. It interferes with respiratory chain and oxidative phosphorylation enzymes[26–28] and causes oxidative injury with subsequent lipid peroxidation, DNA damage, and protein oxidation.[29] The EP4 receptor agonist reduced the number of apoptotic cells in the kidney and its cytoprotective action was responsible for the rapid regeneration of the renal

function. More than 90% of rats survived in the EP4 receptor agonist-treated group, in comparison to 40% in the control group, indicating that the EP4 receptor agonist reduced the severity of ARF and, therefore, could potentially be beneficial in treating ARF. As both the EP2 and EP2/4 receptor agonists had no influence on serum biochemical parameters and the EP2 receptor agonist did not improve the survival of rats with ARF, we suggest that the EP4 receptor is essential for the prevention and/or restoration of the kidney structure and function in ARF. On the contrary, in a model of CRF, all three tested molecules were effective in preserving kidney function suggesting that both EP2 and EP4 receptor agonism is capable of preventing progressive deterioration of the remnant glomerular function in this rat model. CRF is associated with reduction of GFR, elevated serum Cr values, glomerulosclerosis, and tubulointerstitial injury. These are reproduced in the rat remnant kidney model of CRF, where the initial compensatory renal growth response to reduction of the parenchyme is followed in the long term by a progressive renal scarring. Typically, the late renal fibrotic changes are associated with the progressive loss of glomerular cells, tubular atrophy, and their replacement by fibrous tissue.[30]

EP receptors have been shown to be expressed in various kidney cells including mesangial cells.[31] Recently, the role of the EP1 receptor in nephropathy using receptor-specific small molecule inhibitors has been demonstrated.[18] It was suggested that selective inhibition of the EP1 receptor inhibits glomerular hypertrophy and proteinuria, TGF-beta and fibronectin transcriptional activation, preventing the development of diabetic renal injury in rats.[18]

Here, we show in pre-clinical models with impaired renal function that the expression of the EP2 and the EP4 receptor is not significantly regulated at a message level. Interestingly, EP4 receptor agonist is more effective in ARF, whereas EP2, EP4, and EP2/4 receptor agonism is both effective in preventing the progression of the chronic renal failure. Thus, it is suggested that both EP2 and EP4 receptors play a major role in regulating the kidney function. Following Nx, the expression of the EP4 receptor remains similar to sham rats, whereas the expression of the EP2 receptor is downregulated and then upregulated at week 9 following therapy with all three receptor agonists.

Prostaglandins and in particular PGE_2 in the normal kidney physiology regulate vascular smooth muscles, GFR, the release of salt, water re-absorption, and the release of renal hormones.[32] Our results indicate that a role for PGE_2 signaling via the EP2 and EP4 receptor in kidney may be more critical than has been recognized to date. The observed cytoprotection against glomerular degeneration, the preservation of proximal and distal tubule structures, and the presence of newly formed nephrogenic structures seen in EP2 and EP4 receptor agonist-treated kidneys suggest that PGE_2 signaling via the EP2 and EP4 receptor may provide a morphogenic signal for the repair and regeneration of postnatal renal tissue in both the acute and chronic renal failure. PGE_2 is, therefore, not involved only in regulating renal functions but it also has an important role in the repair of damaged renal tissue or in protecting renal tissue against insult and injury.

As the process of the chronic kidney failure in humans occurs over years, delaying the progression is critical for the treatment of chronic kidney diseases. Selective agonists of the EP4 and/or the EP2 receptor, thus, may be used to improve renal architecture during the course of renal disease and recover the lost renal function.

MATERIALS AND METHODS

Screening for PGE_2 selective agonists

Compounds were categorized based on their ability to bind various prostanoid receptors and all three molecules were selective agonists when measured against other PGE_2 receptors such as prostaglandin I (IP) and prostaglandin D (DP) (Paralkar *et al.*,[33]data not shown). EP2 and EP4 receptor agonism was further defined by the ability of compounds to increase intracellular cAMP levels in human embryonic kidney-293 cells overexpressing either the EP2 or the EP4 receptor. cAMP was quantitated using a radioimmunoassay kit according to the manufacturer's instructions (DuPont/NEN Research Products, Boston, MA, USA).

$HgCl_2$ model of ARF

Wistar male rats of 240–250 g were maintained in acclimatized cages on a 12 h light–dark cycle and had free access to standard laboratory chow and tap water. The nephrotoxin (mercury chloride, $HgCl_2$, 2.5 mg/kg) was administered subcutaneously (s.c.) in a bolus upon basal serum biochemistry values and animals were randomly assigned to one of seven groups: (1) $HgCl_2$ control ($n=10$); (2) $HgCl_2$ + CP-043,305-02 (1 mg/kg, $n=10$); (3) $HgCl_2$ + CP-043, 305-22 (10 mg/kg, $n=10$); (4) $HgCl_2$ + CP-044,519-02 (1 mg/kg, $n=10$); (5) $HgCl_2$ + CP-044,519-02 (10 mg/kg, $n=10$); (6) $HgCl_2$ + CP-536,745-01 (1 mg/kg, $n=10$); and (7) $HgCl_2$ + CP-536,745-01 (10 mg/kg, $n=10$). The compound was administered 10 min after $HgCl_2$ injection and continued at 24-h intervals until termination. Control animals received the same volume of vehicle buffer (beta-c-dextran) at the same time points. Animal models were approved by the Institutional Scientific Board.[34]

5/6 nephrectomy model of CRF

Male Wistar rats weighing approximately 400 g were fed standard rat chow *ad libitum* and were given free access to water. A total of 36 animals underwent 5/6 nephrectomy (5/6 Nx).[35] The study followed the previously published protocol.[36] Animals were subjected to unilateral 2/3 nephrectomy (left kidney) under ketamin/diazepam anesthesia (100 or 2.5 μg/kg, respectively). After 2 weeks, the right kidney was surgically removed under anesthesia.[13] In the first experiment, 5/6 Nx rats were randomly assigned into five groups: (1) Sham operated ($n=5$); (2) Nx-control ($n=12$); (3) Nx + CP-536,745-01 (10 mg/kg, 1 ×/week; $n=25$); (4) Nx + CP-043,305-02 (10 mg/kg, 1 ×/week; $n=25$); and (5) Nx + CP-044,519-02 (10 mg/kg, 1 ×/week; $n=25$). At 2 weeks following the second operation, CP-536,745-01, CP-043,305-02, or CP-044,519-02 were dissolved in beta-c-dextran and administered via the rat tail vein once a week throughout the period of 11 weeks. Control animals received only beta-c-dextran as a vehicle. Animals were killed 11 weeks following Nx by overdose of sodium pentobarbital. In the second experiment, 5/6 Nx rats were randomly assigned into three groups: (1) Nx-

control ($n=12$); (2) Nx + CP-044,519-02 (10 mg/kg, 1 ×/week; $n=12$); and (3) Nx + CP-044,519-02 (10 mg/kg, 3 ×/week; $n=12$). Two weeks following the second operation, CP-044,519-02 was dissolved in beta-c-dextran administered via the rat tail vein once or three times a week throughout the period of 11 weeks. Control animals received only beta-c-dextran as a vehicle. Animals were killed 11 weeks following Nx by overdose of sodium pentobarbital.

RNA isolation and gene expression analysis by real-time polymerase chain reaction

Total RNA was isolated from kidneys of sham-operated rats and rats receiving CP-536,745-01, CP-043,305-22, or CP-044,519-02 at 0, 2, 5, 7, and 9 weeks following 5/6 Nx and relative quantification of gene expression was carried out as previously described.[37,38] Beta-actin was chosen as the most stable housekeeping gene for the CP-043,305-02- and CP-044,519-02-treated kidneys and glyceraldehyde-3-phosphate dehydrogenase for the CP-536,745-01 therapy. The following gene-specific primers were used: forward GTG CTG GTA ACG GAA CTG GT and reverse CGT GGC CAG ACT AAA GAA GG for EP2; forward ACA CCA CCT CGC TGA GAA CT and reverse GCT CCC ACT AAC CTC ATC CA for EP4; forward CAT CCT GAA CCG TCT GTG TG and reverse TTT CCA CCA AGG ACC CAC TA for albumin; forward ATG ATT CTA CCC ACG GCA AG and reverse CTG GAA GAT GGT GAT GGG TT for glyceraldehyde-3-phosphate dehydrogenase, and forward GGG AAA TCG TGC GTG ACA TT and reverse GCG GCA GTG GCC ATC TC for beta-actin.

Renal function

$HgCl_2$ model of ARF. Blood samples (0.5 ml) were obtained from the orbital venous plexus at 0, 24, 48, 72, and 128 h after $HgCl_2$ administration. Serum creatinine was measured by the Jaffe method (alkaline picrate) and blood urea nitrogen by enzymatic glutamate dehydrogenase-UV procedure as previously described in Vukicevic *et al.*[39] The cumulative survival rate was also observed and recorded for both control and experimental rats. Animals were killed 6 days after $HgCl_2$ treatment.

5/6 nephrectomy model of CRF. Blood samples and 24-h urine collections in metabolic cages were taken on weeks 2, 5, 8, and 11. Serum and urine creatinine was measured using the standard Jaffé method. The GFR was determined using serum creatinine over urine creatinine as adjusted to body weights.

Histology

$HgCl_2$ model of ARF. At termination, kidneys were removed, cut longitudinally and one half of each kidney was fixed in 4% paraformaldehyde, embedded in paraffin, and 4 μm paraffin sections were cut with a microtome and stained with periodic acid-Schiff stain. Qualitative and semiquantitative assessments of the level of necrosis of proximal tubules and other pathological changes in kidney sections were made blindly using a light microscope. The level or severity of necrosis in proximal tubules was ranked for each animal on a scale of 0–12, where 0 represents no cellular necrosis and 12 represents a level of necrosis that involves virtually every proximal tubular segment visible in the plane of section as previously described.[40]

5/6 nephrectomy model of CRF. Kidneys for histological examination were fixed in 2% paraformaldehide and 7 μm paraffin sections were cut and stained with hematoxylin and eosin. Tubulointerstial injury, defined as tubular dilatation and/or atrophy, interstitial fibrosis, and inflammatory cell infiltrate, as well as glomerular damage, were graded using a semiquantitative scale from 0 to 4 according to the following criteria: 0 = no changes; 1 = focal changes involving 1–25% of the sample; 2 = changes affecting 26–50% of the sample; 3 = changes involving 51–75% of the sample, and 4 = lesions affecting more than 75% of the sample.[41] Two independent observers performed histological studies in a blinded fashion.

Immunohistochemical analyses. Immunocytochemistry was performed using the immunoperoxidase detection system (Zymed, San Francisco, CA, USA). The following monoclonal antibodies were used: PCNA (DAKO, Copenhagen, Denmark), smooth muscle α-actin (DAKO), intracellular adhesion molecule (CD 54; DAKO), and Mac (CD-68) (DAKO). A minimum of 3000 cells was counted per kidney section stained for PCNA and smooth muscle α-actin, and the number of positive cells was expressed as a percentage of total counted cells in subdivisions of cortex, and/or the S3 zone.

Apoptosis

$HgCl_2$ model of ARF. Apoptotic cells were detected by a TACS 2 TdT *in situ* apoptosis system (Trevigen, Gaithersburg, MD, USA), which detects double-strand breaks in genomic DNA by enzymatic incorporation of a biotinylated nucleotide with terminal deoxynucleotidyl transferase followed by binding of streptavidin-horseradish peroxidase. The modifications of the procedure include labeling time of 90 min, coverslips in all reactions to prevent drying of samples, and incubation time of streptavidin–HRP of 15 min.[42]

Statistical analysis

All data are presented as mean ± s.e.m. One-way analysis of variance was performed to determine the effect of treatment on biochemical parameters. Student *t*-test was used to determine the effect of therapy on the gene expression level as compared to sham animals. Statistical evaluation of the survival rate was carried out by the Petö-Wilcoxon test, and results were considered significant when the probability of error (P) was <0.05.

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